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(54) Title: NEPOVIRUS RESISTANCE IN GRAPEVINE

## (57) Abstract

In general, the invention features a method for selecting a transgenic grapevine or grapevine component having increased resistance to a fanleaf disease, the method including the steps of: (a) transforming a grape plant cell with a grape nepovirus coat protein nucleic acid molecule or fragment thereof which is capable of being expressed in the plant cell; (b) regenerating a transgenic grapevine or grapevine component from the plant cell; and (c) selecting a transgenic grapevine or grapevine component which expresses, at a low level, the nucleic acid molecule or fragment thereof, wherein the low level expression increases the resistance of the transgenic grapevine or grapevine component to fanleaf disease. The invention also relates to an isolated nucleic acid molecule encoding a coat protein or fragment thereof of a "Geneva" isolate of a grapevine nepovirus virus.

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NEPOVIRUS RESISTANCE IN GRAPEVINEBackground of the Invention

5        This invention relates to disease resistance in plants.

Grapevine fanleaf virus (GFLV) is a grape nepovirus, which is transmitted from plant to plant by the dagger nematode, *Xiphinema index*. GFLV is the agent responsible for grapevine fanleaf disease, which occurs worldwide. The disease is named for the fan-leaf shaped appearance of GFLV-infected leaves. It is one of the most

10      damaging and widespread diseases of grapevine. Symptoms of GFLV infection include abnormal shoot morphology and discolorations of the leaves, yielding a fan-like appearance (Agrios, *Plant Pathology*, 3<sup>rd</sup> Edition, Academic Press, 1988, pp. 687-688). In addition, fruit production of infected vines is low, with grapevines producing small bunches having abnormal fruit set and ripening. Ultimately, infected grapevines

15      degenerate and die.

Long range spread of GFLV is believed to be by use of infected planting material. While the natural host range is thought to be restricted to grape, GFLV is also transmissible to a wide range of herbaceous species by sap-rubbing inoculation.

20      *Chenopodium quinoa* is a useful diagnostic species for the virus. In general, GFLV isolates are antigenically uniform and diagnosis by ELISA is a standard procedure.

Current strategies for controlling grapevine fanleaf disease and other nepovirus-induced diseases in vineyards include nematode control (for example, soil fumigation and use of other pesticides), breeding rootstocks for resistance to nematode feeding, breeding grapevines for resistance to GFLV, and planting certified disease-free grapevines.

Summary of the Invention

In general, the invention features a method for producing and selecting a transgenic grapevine or grapevine component having increased resistance to a fanleaf disease. The method generally involves: (a) transforming a grape plant cell with a grape

30      nepovirus coat protein nucleic acid molecule or fragment thereof (for example, a grape

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nepovirus coat protein nucleic acid molecule or fragment thereof having about 50% or greater sequence identity to SEQ ID NO: 1) which is capable of being expressed in a plant cell; (b) regenerating a transgenic grapevine or grapevine component from the plant cell; and (c) selecting a transgenic grapevine or grapevine component which expresses, at 5 a low level, the nucleic acid molecule or fragment thereof, wherein the low level expression increases the resistance of the transgenic grapevine or grapevine component to fanleaf disease as compared to plants expressing the nucleic acid molecule at a high level. Low level expression of the grape nepovirus mRNA or of the expressed coat protein itself in the transgenic plant is measured according to standard methods including, without 10 limitation, Northern blot analysis, ELISA, and inoculation of transgenic plants with virus and selection of resistant vines. In preferred embodiments, the nucleic acid molecule or fragment thereof is encoded by a transgene found in the transgenic grapevine. In other preferred embodiments, the nucleic acid molecule or fragment thereof is expressed as in a sense or antisense orientation. In yet other preferred embodiments, such grape nepovirus 15 coat protein nucleic acid molecules or fragments thereof (a non-limiting example being a sense nontranslatable grape nepovirus viral coat protein mRNA having an out-of-reading frame initiation ATG initiation codon with the remainder of the mRNA being out of frame) is expressed in the transgenic grapevine or grapevine component.

As is discussed above, the invention also includes fragments of a grape 20 nepovirus coat protein nucleic acid molecule that facilitate, when expressed at low levels, an increased resistance of a transgenic grapevine or grapevine component thereof, to a fanleaf disease. Thus, grape nepovirus coat protein nucleic acid sequences described herein or portions thereof may be expressed in a plant to facilitate disease resistance. Sequences that mediate an increased resistance to a fanleaf disease are considered useful 25 in the invention. As used herein, the term "fragment," as applied to sequences of a nucleic acid molecule, means at least 5 contiguous nucleotides, preferably at least 10 contiguous nucleotides, more preferably at least 20 to 30 contiguous nucleotides, and most preferably at least 40 to 80 or more contiguous nucleotides. Fragments of a grape nepovirus nucleic acid molecule can be produced and, subsequently, integrated into any 30 standard expression vector (for example, those described herein) according to methods known to those skilled in the art.

Preferably, the grapevine useful in the invention is a member of the genus *Vitis*; and the grapevine component is a somatic embryo, a scion, a rootstock, or a mother block. In still other preferred embodiments, the fanleaf disease is grapevine fanleaf disease caused by a grape nepovirus. In yet other preferred embodiments, the grape nepovirus is a grapevine fanleaf virus or an arabis mosaic virus.

In another aspect, the invention features a vineyard including three or more transgenic grapevines or grapevine components each of which express, at a low level, a grape nepovirus coat protein nucleic acid molecule or fragment thereof, wherein the low level expression of the nucleic acid molecule or fragment thereof increases resistance of 10 the transgenic grapevines or grapevine components in the vineyard to fanleaf disease.

In still another aspect, the invention features a substantially pure protein (for example, a recombinant protein) including an amino acid sequence having at least 97% amino acid identity to the amino acid sequence of the 'Geneva' isolate grape nepovirus coat protein shown in Fig. 1 (SEQ ID NO: 2). In preferred embodiments, the protein 15 includes the amino acid sequence of the grape nepovirus coat protein shown in Fig. 1 (SEQ ID NO: 2). In yet other preferred embodiments, the protein has the amino acid sequence of the grape nepovirus coat protein shown in Fig. 1 (SEQ ID NO: 2) or fragments thereof.

In yet another aspect, the invention features an isolated nucleic acid molecule 20 encoding a protein (for example, a recombinant protein) including an amino acid sequence having at least 97% amino acid identity to the amino acid sequence of the 'Geneva' isolate grape nepovirus coat protein shown in Fig. 1 (SEQ ID NO: 2). In preferred embodiments, the protein encoded by the nucleic acid molecule includes the amino acid sequence of SEQ ID NO: 2. In yet other preferred embodiments, the protein 25 encoded by the nucleic acid molecule has the amino acid sequence of SEQ ID NO: 2 or a fragment thereof.

In another aspect, the invention features an isolated nucleic acid molecule (for example, a DNA molecule) that encodes a grape nepovirus coat protein that specifically hybridizes to a nucleic acid molecule that includes the nucleic acid sequence of Fig. 1 30 (SEQ ID NO: 1). Preferably, the specifically hybridizing nucleic acid molecule encodes a grape nepovirus sequence that mediates resistance when expressed at low levels in a

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grape plant cell to a fanleaf disease (for example, grapevine fanleaf disease). The invention also features an RNA transcript having a sequence complementary to any of the isolated nucleic acid molecules described above.

In related aspects, the invention further features a cell (for example, a prokaryotic cell or a eukaryotic cell such a mammalian cell or yeast cell) which includes an isolated nucleic acid molecule of the invention. In preferred embodiments, the cell is a bacterium (for example, *E. coli* or *Agrobacterium tumefaciens*) or is a plant cell (for example, a grape plant cell from any of the cultivars listed herein). Such a plant cell has resistance against a fanleaf disease (for example, grapevine fanleaf disease).

10 In still other related aspects, the invention further features a vector (for example, a plant expression vector) which includes an isolated nucleic acid molecule of the invention. In a preferred embodiment, the isolated nucleic acid molecule is operably linked to an expression control region that mediates expression of a protein encoded by the nucleic acid molecule (for example, a nucleic acid molecule (such as DNA) expressed as a sense translatable or a sense nontranslatable mRNA transcript, or as an antisense mRNA transcript).

15 In still other aspects, the invention features a transgenic plant or plant component (for example, a grapevine or grapevine component) that includes a nucleic acid molecule encoding a protein (for example, a recombinant protein) encoding an amino acid sequence having at least 97% amino acid identity to the amino acid sequence of the 'Geneva' isolate grape nepovirus coat protein shown in Fig. 1 (SEQ ID NO: 2). In preferred embodiments, such a transgenic plant or plant component includes a nucleic acid molecule of SEQ ID NO: 1. Moreover, fragments of these sequences may be made such that the nucleic acid molecule expresses a sense translatable, sense nontranslatable, or antisense RNA transcript. In still other preferred embodiments, the plant or plant component has the nucleotide sequence of SEQ ID NO: 1 or fragments thereof. Such plants or plant components which include the nucleic acid molecules of the invention have an increased level of resistance against a fanleaf disease caused by a grape nepovirus (for example, GFLV).

20 30 The methods and GFLV sequences described herein are useful for providing disease resistance or tolerance or both on a variety of grapevines (for example, *Vitis spp.*,

*Vitis spp.* hybrids, and all members of the subgenera *Euvitis* and *Muscadinia*), including scion or rootstock cultivars. Exemplary scion cultivars include, without limitation, those which are referred to as table or raisin grapes and those used in wine production such as Cabernet Franc, Cabernet Sauvignon, Chardonnay (for example, CH 01, CH 02, CH 5 Dijon), Merlot, Pinot Noir (PN, PN Dijon), Semillon, White Riesling, Lambrusco, Thompson Seedless, Autumn Seedless, Niagrara Seedless, and Seval Blanc. Rootstock cultivars that are useful in the invention include, without limitation, *Vitis rupestris* Constantia, *Vitis rupestris* St. George, *Vitis californica*, *Vitis girdiana*, *Vitis rotundifolia*, *Vitis rotundifolia* Carlos, Richter 110 (*Vitis berlandieri* x *rupestris*), 101-14 Millardet et 10 de Grasset (*Vitis riparia* x *rupestris*), Teleki 5C (*Vitis berlandieri* x *riparia*), 3309 Courderc (*Vitis riparia* x *rupestris*), Riparia Gloire de Montpellier (*Vitis riparia*), 5BB Teleki (selection Kober, *Vitis berlandieri* x *riparia*), SO<sub>4</sub> (*Vitis berlandieri* x *rupestris*), 41B Millardet (*Vitis vinifera* x *berlandieri*), and 039-16 (*Vitis vinifera* x *Muscadinia*).

15 The invention also features scions, rootstocks, somatic or zygotic embryos, cells, or seeds that are produced from any of the transgenic grapevines or grapevine components described herein.

By "nontranslatable" is meant an mRNA sequence that is not translated into a protein. Examples of such nontranslatable sequences include, without limitation, 20 sequences including an initiation ATG codon followed by an engineered frameshift mutation and stop codon to prevent translation of the mRNA into a protein. Grape nepovirus coat protein genes expressing such nontranslatable mRNA sequences may be constructed according to standard methods (for example, those described herein).

By "low level expression" is meant a level of grape nepovirus coat protein 25 gene expression in a transgenic plant that is greater than zero and that is sufficiently low to impart fanleaf disease resistance. "High level expression" refers to the level of gene expression found in a transgenic plant expressing a coat protein gene that is too high to confer resistance to the disease. Exemplary methods for analyzing low level expression of a grape nepovirus coat protein gene includes, without limitation, Northern blot analysis 30 for detection of a mRNA transcript, as well as immunological techniques such as ELISA for detection of a protein.

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By "substantially identical" is meant a protein or nucleic acid molecule exhibiting at least 97%, and preferably 98%, or most preferably 99% identity to a reference amino acid sequence (for example, the amino acid sequence shown in Fig. 1; SEQ ID NO: 2) or nucleic acid sequence (for example, the nucleic acid sequences shown 5 in Fig. 1; SEQ ID NO: 1). For proteins, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids or greater. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 10 nucleotides or greater.

Sequence identity, at the amino acid or nucleic acid levels, is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705, BLAST, or 15 PILEUP/Prettybox programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative amino acid substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and 20 phenylalanine, tyrosine.

By a "substantially pure protein" is meant a grape nepovirus coat protein (for example, the coat protein from the Geneva, N.Y. grape nepovirus isolate (Fig. 1; SEQ ID NO: 2)) that has been separated from components which naturally accompany it. Typically, the protein is substantially pure when it is at least 60%, by weight, free from 25 the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, protein. A substantially pure protein (for example, the coat protein of the 'Geneva' grape nepovirus isolate) may be obtained, for example, by extraction from a natural source (for example, a GFLV-CP infected plant 30 such as *C. quinoa*); by expression of a recombinant nucleic acid encoding a protein; or by chemically synthesizing the protein. Purity can be measured by any appropriate method,

for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

By "isolated nucleic acid molecule" is meant a nucleic acid molecule (for example, DNA) that is free of the nucleic acids which, in the naturally-occurring genome 5 of the organism from which the nucleic acid molecule of the invention is derived, flank the nucleic acid molecule. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or restriction 10 endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional protein sequence.

By "specifically hybridizes" is meant that a nucleic acid molecule that is capable of hybridizing to a nucleic acid sequence (for example, DNA) at least under low stringency conditions, and preferably under high stringency conditions.

15 By "protein" is meant any chain of amino acids, including polypeptides, regardless of length or post-translational modification (for example, glycosylation or phosphorylation), including polypeptides.

By "positioned for expression" is meant that the nucleic acid molecule (for example, DNA) is positioned adjacent to a sequence which directs transcription of the 20 nucleic acid molecule (for example, a gene expressing a nontranslatable antisense sequence or sense nontranslatable sequence).

By "expression control region" is meant any minimal sequence sufficient to direct transcription. Included in the invention are promoter and enhancer elements that are sufficient to render promoter-dependent gene expression controllable for cell-, tissue-, 25 or organ-specific gene expression, or elements that are inducible by external signals or agents (for example, light-, pathogen-, wound-, stress- or hormone-inducible elements; or constitutive elements); such elements may be located in the 5' or 3' regions of the native gene or engineered into a transgene construct.

30 By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way to permit gene expression when the appropriate molecules (for example, transcriptional activator proteins) are bound to the regulatory sequence(s).

By "plant cell" is meant any self-propagating cell bounded by a semi-permeable membrane and containing a plastid. A plant cell, as used herein, is obtained from, without limitation, seeds, suspension cultures, embryos, meristematic regions, callus tissue, protoplasts, leaves, roots, shoots, somatic and zygotic embryos, as well as  
5 any part of a reproductive or vegetative tissue or organ.

By "plant component" is meant a part, segment, or organ obtained from an intact plant or plant cell. Exemplary plant components include, without limitation, somatic embryos, leaves, fruits, scions and rootstocks.

By "vineyard" is meant a plot of land which includes three or more transgenic  
10 grapevines or grapevine components which were selected for low level expression of a grape nepovirus coat protein nucleic acid molecule or fragment thereof.

By "transgenic" is meant any cell which includes a nucleic acid molecule (for example, a DNA sequence) which is inserted by artifice into a cell and becomes part of the genome of the organism (in either an integrated or extrachromosomal fashion for  
15 example, a viral expression construct which includes a subgenomic promoter) which develops from that cell. As used herein, the transgenic organisms are generally transgenic grapevines or grapevine components and the nucleic acid molecule (for example, a transgene) is inserted by artifice into the nuclear or plastidic compartments of the plant cell. Preferably, such transgenic grapevine or grapevine component express at least one  
20 sense translatable, sense nontranslatable, or antisense grape nepovirus transcript (for example, a GFLV-CP antisense sequence).

By "transgene" is meant any piece of a nucleic acid molecule (for example, DNA) which is inserted by artifice into a cell, and becomes part of the organism (integrated into the genome or maintained extrachromosomally) which develops from that  
25 cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

By "antisense nucleic acid sequence" is meant a nucleotide sequence that is complementary to a transcribed RNA. In general, such an antisense sequence will usually  
30 be at least 15 nucleotides, preferably about 15-200 nucleotides, and more preferably 200-2,000 nucleotides in length. The antisense sequence may be complementary to all or a

portion of the transcribed RNA nucleotide sequence (for example, a grape nepovirus sequence such as the GFLV-CP antisense constructs described herein or any of the GFLV sequences described in Brandt et al., *Arch. Virol.* 140: 157-164, 1995; Margis et al., *J. Gen. Virol.* 74: 1919-1926, 1993; Fuchs et al., *J. Gen Virol.* 955-962, 1989; Serghini et al., *J. Gen. Virol.* 71: 1433-1441, 1990; Bardonnet et al., *Plant Cell Reports* 13: 357-360, 1994; Krastanova et al., *Plant Cell Rep.* 14:550-554, 1995; Ritzenthaler et al., *J. Gen. Virol.* 72: 2357-2365, 1991; Mauro et al., *Plant Science*: 112: 97-106, 1995; and Sanchez et al., *Nucleic Acids. Res.* 19: 5440, 1992), and, as appreciated by those skilled in the art, the particular site or sites to which the antisense sequence binds as well as the length of the antisense sequence will vary, depending upon the degree of inhibition desired and the uniqueness of the antisense sequence. Preferably, a transcriptional construct expressing a grape nepovirus sequence (for example, a GFLV-CP antisense nucleotide sequence) includes, in the direction of transcription, an expression control region, the sequence coding for the antisense RNA on the sense strand, and a transcriptional termination region. Antisense grape nepovirus sequences (for example, GFLV sequences) may be constructed and expressed as described herein or as described, for example, in van der Krol et al., *Gene* 72: 45, 1988; Rodermel et al., *Cell* 55: 673, 1988; Mol et al., *FEBS Lett.* 268: 427, 1990; Weigel and Nilsson, *Nature* 377: 495, 1995; Cheung et al., *Cell* 82: 383, 1995); and U.S. Pat. No. 5,107,065.

By "increased resistance to a fanleaf disease" is meant a greater level of resistance to a fanleaf disease (for example, any disease caused by a grape nepovirus such as those caused by GFLV, arabis mosaic virus, and the like) in a transgenic grapevine (or grapevine component or cell or seed thereof) than the level of resistance relative to a control grapevine (for example, a non-transgenic grapevine). In preferred embodiments, the level of resistance in a transgenic grapevine is at least 5 to 10% (and preferably 20%, 30%, or 40%) greater than the resistance of a control grapevine. In other preferred embodiments, the level of resistance to fanleaf disease is 50% greater, 60% greater, and more preferably even 75% or 90% greater than a control grapevine; with up to 100% resistance as compared to a control grapevine being most preferred. The level of resistance is measured using conventional methods. For example, the level of resistance to fanleaf disease may be determined by comparing physical features and characteristics

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- (for example, plant height and weight, or by comparing disease symptoms, for example, delayed lesion development, reduced lesion size, leaf wilting and curling, mottling and necrosis of leaves, deformity of canes, number of internodes, mosiac rings on leaves, and discoloration of cells) of transgenic grapevines. Infectivity of a grape nepovirus (for 5 example, a GFLV or an arabis mosaic virus) can also be monitored using, for example, standard ELISA.

As is discussed above, it has been discovered that the low level expression of a grape nepovirus sense-translatable coat protein gene, as well as an antisense sequence, provides transgenic grapevines with resistance against disease caused by a grape 10 nepovirus. Accordingly, the invention provides a number of important advances and advantages for viticulturists. For example, by selecting transgenic grapevines which express low levels of a recombinant grape nepovirus coat protein gene and thus have increased resistance against grape nepovirus infection, the invention facilitates an effective and economical means for protection against grapevine fanleaf disease and other 15 grape nepovirus-induced diseases. Such protection reduces or minimizes the need for traditional chemical practices (for example, soil fumigation) typically used by viticulturists for controlling the spread of a grape nepovirus and provides protection against these disease-causing pathogens. In addition, because grape plants expressing such grape nepovirus sequences are less vulnerable to grape nepovirus infection and 20 fanleaf disease, the invention further provides for increased production efficiency, as well as for improvements in quality, color, flavor, and yield of grapes. Furthermore, because the invention reduces the necessity for chemical protection against grapevine pathogens, the invention also benefits the environment where the vineyards are planted. The 25 invention may also be used in combination with cultivated rootstocks having resistance to soil-borne nematodes.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

#### Detailed Description

The drawings will first be described.

30 **Drawings**

**Fig. 1** is a schematic illustration showing the nucleotide (SEQ ID NO: 1) and

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deduced amino acid sequence (SEQ ID NO: 2) of the coat protein of a Geneva, N.Y. grape nepovirus isolate.

Fig. 2 is a schematic illustration showing the maps of the plant expression vectors containing different viral gene constructs.

5 Fig. 3 shows the results of experiments analyzing the expression levels of grapevine fanleaf coat protein in transgenic 3309C and Gloire.

A description for the production of disease resistant transgenic grapevines now follows. Transgenic grape plants expressing either sense translatable, sense nontranslatable, or antisense sequences of grapevine fanleaf virus coat protein (GFLV-  
10 CP) genes were regenerated from embryogenic callus cultures derived from anthers of rootstocks (3309 Couderc ("3309C"), Riparia Gloire ("Gloire"), Teleki 5C ("5C"), 110 Richter ("110R"), SO4, and MGT 101-14 ("101-14")). Unexpectedly, transgenic plants expressing low levels of a recombinant grapevine fanleaf coat protein gene were found to be resistant to fanleaf disease.  
15 The examples provided below are for the purpose of illustrating the invention, and should not be construed as limiting.

### Results

#### Initiation of Embryogenic Callus and Embryogenesis

Callus was initiated from grape cultivars: Gloire, 3309C, 5C, 110R, and 101-  
20 14 on MSE medium (*infra*). Anthers from flower buds of the five rootstocks began to swell after one week in culture. After four weeks, a smooth, gelatinous, bright yellow callus developed. At this time, some embryos of Gloire, 3309C, and 110R were visible on the callus tissue. After eight weeks, all the calli were transferred to HMG medium (*infra*) to permit further development of the embryos. By the eighth week on HMG  
25 medium, many embryo clusters were induced from the callus tissue.

#### Plant Regeneration

After cultivation for eight to sixteen weeks in HMG medium, embryo clusters, and hypocotyls were found to develop from the calli. At the same time, secondary embryos were continually produced from the primary embryos. Embryo clusters were  
30 next transferred to MGC medium (*infra*) to increase embryo size and growth rate.

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However, fewer embryos were produced on MGC medium as compared to HMG medium for all rootstock cultivars that were examined. Embryo development of cultivar 110R was found to be dependent on the use of both media; HMG medium was required to induce many small secondary embryos and MGC medium was needed to simulate 5 hypocotyl growth.

Hypocotyls were subsequently transferred onto a woody plant medium (Lloyd and McCown, *infra*) and shoots appeared within one to two months. The plantlets were generally induced at thirty to sixty-six frequency on woody plant medium.

10 The resulting plantlets were then transplanted to soil and kept in the greenhouse. Plants of Gloire, 5C, 110R, 101-14, and 3309C exhibited normal morphology.

#### Maintenance of Somatic Embryogenesis

A continuous supply of embryogenic calli were produced using an embryo cycling method; pieces of hypocotyl induced embryogenic callus on the MSE medium in 15 two to three months. These calli were amenable for transformation because they developed many uniform embryos. Embryos of 5C required culture on MSE medium for three months, followed by culture on HMG medium for two to three months to induce the formation of embryos. The duration of time required for embryo cycling (embryogenic callus to hypocotyl and back to embryogenic callus) varied for the different cultivars; 20 Riparia Gloire required two to three months, 3309C and 101-14 required five to six months, and 5C required six to seven months.

#### Transformation

Using standard techniques of molecular biology, a nucleotide sequence 25 encoding a coat protein gene of a Geneva, NY grape nepovirus isolate was isolated and characterized. The nucleic acid sequence (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) of the 'Geneva' isolate coat protein gene are shown in Fig. 1. These sequences were also compared to three different GFLV isolates from France (Sergolini et al., *J. Gen. Virol.* 71: 1433-1442, 1990), California (Sanchez et al., *Nucleic Acids Res.* 19 5440, 1991), and Austria (Brandt et al., *Arch. Virol.* 140: 157-164, 1995) 30 using the Prettybox program. The percentage of identity of the amino acid sequences of the French, Californian, and Austrian isolates with the 'Geneva' isolate was 96.4%,

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95.0%, and 95.4%, respectively.

Three different gene constructs (sense translatable, sense nontranslatable, and antisense) were used to transform grape (Fig. 2). Small somatic embryos and embryogenic calli of the five rootstocks Gloire, 3309C, 110R, 5C, and 101-14 were

- 5 cocultivated with *A. tumefaciens* strain C58Z707 harboring binary vectors carrying the coat protein gene of the 'Geneva' isolate. After cocultivation, somatic embryos were transferred to HMG or MSE media with cefotaxime, carbenicillin, and kanamycin to select transgenic embryos and plants. Transgenic plants were thus generated.

#### Analysis of Transgenic Plants

- 10 In an experiment with a GFLV-CP sense-translatable and  $\beta$ -glucuronidase gene expression constructs (FLcpST+GUS, Fig. 2), putative transgenic plants of Gloire and of 110R were assayed for GUS activity, the NPTII gene, and expression of the GFLV-CP gene by ELISA. By PCR analysis, about 93% of the transformed Gloire plants were found to have the GFLV-CP gene of the expected size, yet these plants were  
15 negative for GFLV expression by ELISA.

- These results indicated that GFLV-CP gene expression in the Gloire transgenics was too low to detect using ELISA. In contrast cultivar 3309C was transformed with GFLV-CP sense-translatable construct in a vector without GUS. We analyzed coat protein expression of putative transgenic plants, and found that 97.7%  
20 ELISA positive plants. Among these plants, 37.7% showed low expression ( $0.1 < OD_{405} > 0.5$ ), 30.7% showed medium expression ( $0.5 < OD_{405} > 1.0$ ), and 31.0% had high expression ( $OD_{405} > 1.0$ ). Nontransformed control plants were negative  
( $OD_{405} < 0.020$ ).

- In an another series of experiments, ELISA results also revealed different  
25 levels of expression of GFLV coat protein gene in transgenic plants of 3309C and Gloire. Low coat protein gene expression was observed in 61% and 53% of the transformed 3309C and Gloire, respectively. Medium coat protein gene expression was found in 26% and 22%, respectively. High coat protein gene expression was found in 13% and 25%, respectively, of the transformed 3309C and Gloire (Fig. 3).

#### Protection Against GFLV Infection

Transgenic plants were tested for GFLV resistance as follows. Plants were

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inoculated with GFLV by heterografting to GFLV-infected *C. quinoa* or by grafting to nontransgenic GFLV-infected cultivars according to standard methods. The plants were maintained for several months after inoculation and then were evaluated for disease resistance. Disease resistance was assessed by standard ELISA. The results of these 5 experiments are shown in Tables I-V (below). In particular, as is shown in Table III, one transgenic line expressing the antisense expression constructs GFLVcpAntiS (Fig. 2) was found to resist GFLV infection.

**Table I**  
**ELISA Evaluation of Transgenic 110 Richter Heterografted with Infected *C. quinoa***

	Transgenic lines <sup>1</sup>	ELISA 4/31/97	ELISA 5/27/97	ELISA 8/6/97	ELISA 2/12/97	(%) infected
10	#2	0/2	1/2	1/4	1/4	25.0
	#3	0/6	1/6	4/10	4/10	40.0
	#6	0/2	nt	1/2	1/2	50.0
15	#8	0/2	2/2	2/5	NT	40.0
	#21	0/8	nt	8/8	8/8	100.0
	#41	nt	2/8	5/8	NT	62.0
	#45	-	-	2/2	NT	100.0
20	#50	-	3/4	4/4	4/4	100.0
	#84	-	4/6	5/6	5/6	83.0
	Control	-	6/8	14/16	14/16	88.0

<sup>1</sup> FLcpST+GUS (Fig. 2); NT: Not tested

**Table II**  
**ELISA Evaluation of Transgenic *Riparia Gloire* Heterografted with GFLV Infected *C. quinoa***

	Transgenic lines <sup>1</sup>	2 months	4-5 months	Total	(%) infected
5	#5	0/3	0/10	0/10	0
	#6	0/3	0/3	0/3	0
	#7	0/5	2/7	2/7	29.0
	#9	0/5	1/8	1/8	13.0
	#10	1/16	4/14	4/14	29.0
10	#12	0/4	0/4	0/4	0
	#13	3/11	3/8	3/8	38.0
	#14	0/7	0/7	0/7	0
	#15	0/4	11/15	11/15	73.0
	#16	0/5	0/9	0/9	0
15	#19	nt	0/4	0/4	0
	#20	2/12	2/12	2/12	17.0
	#21	0/11	3/14	3/14	14.0
	#22	0/1	0/4	0/4	0
	#B-6	nt	0/8	0/8	0
20	# B-17	nt	6/13	6/13	46.0
	# B-20	0/7	0/6	0/6	0
	# B-24	nt	0/3	0/3	0
	# B-42	0/4	0/4	0/4	0
	Control	23/26	23/26	23/26	88.0

<sup>1</sup> FLcpST+GUS (Fig. 2)

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**Table III**  
**ELISA Evaluation of Transgenic MGT-101-14 Heterografted with Infected *C. quinoa***

	Transgenic lines	ELISA 3/27/97	ELISA 4/31/97	ELISA 5/27/97	ELISA 8/6/97	(%) infected
5	T18,FL(5-1)1 <sup>1</sup>	0/7	nt	0/7	0/7	0
	T18,FL(1-1)1 <sup>2</sup>	0/8	1/8	2/8	4/8	50.0
	T18,FL(1-1)2 <sup>2</sup>	3/4	3/4	3/4	3/4	75.0
	T18,FL(1-1)3 <sup>2</sup>	2/5	3/5	4/5	4/5	80.0
10	T18,FL(1-1)E <sup>2</sup>	0/4	1/4	3/4	4/4	100.0
	T18,FL(B-8) <sup>2</sup>	0/2	1/2	2/2	2/2	100.0
	T18,FL(A-3) <sup>2</sup>	nt	0/4	3/4	4/4	100.0
	T18,FL(K-1) <sup>2</sup>	nt	2/8	2/8	2/8	25.0
	CONTROL	-	-	11/16	23/29	79

15 <sup>1</sup>FLcpAntiS (Fig. 2); <sup>2</sup>FLcpST (Fig. 2)

**Table IV**  
**Micrografting *In vitro***

	Transgenic L./ GFLV grape	Number of plantlets in GH	ELISA 11/27/97	ELISA 1/30/97	ELISA 4/24/97
		6			
20	Riparia # 16/Cabernet sauv.	5	0/2	0/2	0/2
	Riparia # 21/Cabernet sauv.	5	1/5	1/5	5/5
	Riparia # 23/Cabernet sauv.	5	2/5	2/5	4/5
	Riparia # 19/Cabernet sauv.	4	0/4	0/4	0/4
	Riparia B-2/Cabernet sauv.	4	0/4	0/4	0/4
25	Riparia B-13/Cabernet sauv.	4	1/4	2/4	3/4
	Riparia B-17/Cabernet sauv.	2	1/2	1/2	2/2
	Riparia B-67/Cabernet sauv.	2	0/2	0/2	0/2
	Riparia, ht/Cab	5	4/5	4/5	4/4

**Table V**  
**Micrografting *In vitro***

	Transgenic L./ GFLV grape	Number of plantlets in GH	ELISA 11/27/97	ELISA 1/30/97	ELISA 4/24/97
30	Richter 45/Rupestris	3	2/3	2/3	2/3
	Richter 26/Rupestris	6	0/6	0/6	0/6
35	Richter 56/Rupestris	2	0/2	0/2	0/2
	Richter 11/Rupestris	3	0/3	0/3	0/3
	Richter 75/Rupestris	6	2/3	2/3	2/4
	Rupestris/Richter 56	2	0/2	0/2	0/2
	Rupestris/Richter 75	6	2/3	2/3	2/5
40	Rupestris/Richter 4	4	0/3	0/2	0/2
	Richter ht/Rup	4	3/4	3/4	4/4

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Materials and Methods

The above-described results were carried out using the following materials and methods.

Plant materials

5       The rootstock cultivars Couderc 3309 ("3309C") (*V. riparia* x *V. rupestris*),  
Riparia Gloire ("Gloire") (*V. riparia*), Teleki 5C ("5C") (*V. berlandieri* x *V. riparia*),  
MGT 101-14 ("101-14") (*V. riparia* x *V. rupestris*) and 110 Richter ("110R") (*V.*  
*rupestris* x *V. berlandieri*) were used in the above-described experiments. Callus cultures  
were initiated from anthers using the methods of Rajasekaram and Mullins (*J. Exp. Bot.*  
10      30: 399-407, 1979). Flower buds of 3309C, 5C, 110R and 101-14 were collected from a  
vineyard at the Geneva Experiment Station, Geneva, N.Y. Gloire dormant canes were  
collected from the same vineyard and stored in moist perlite in plastic bags at 4°C. Two  
to five node sections were rooted in pots with perlite in the greenhouse; floral buds  
developed within four weeks. Flower buds were harvested prior to anthesis from field-  
15     grown vines. Buds were removed from the clusters and surface sterilized in 70% ETOH  
for one to two minutes. The buds were transferred to 1% sodium hypochlorite for fifteen  
minutes, then rinsed three times in sterile double-distilled water. Anthers were excised  
aseptically from flower buds while using a stereo microscope. To determine which state  
was most favorable for callus induction, the pollen was crushed on a microscope under a  
20     coverslip with a drop of acetocarmine to observe the cytological stage according to  
standard methods.

Media

Four different solid media were used to produce embryos and regenerate plants.

25     The four media used are as follows. (1) Initiation medium. This medium was an  
amended MS medium (Murashige and Skoog, *Physiol. Plant.* 15: 473-497, 1962) and is  
referred to as MSE (Mozsar and Sule, *Vitis* 33: 245-246, 1994). (2) Differentiation  
medium. This medium is referred to as HMG medium as described by Mozsar and Sule  
(*Vitis* 33: 245-246, 1994); (3) Regeneration medium. This medium is referred to as  
30     MGC medium. It is composed of full-strength MS salts amended with 20 g/L sucrose,

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4.6 g/L glycerol, 1 g/L casein hydrolysate and 0.8% Noble agar; and (4) Rooting medium. This medium (pH 5.8) is woody plant medium (Lloyd and McCown, *Proc. Intl. Plant Prop. Soc.* 30: 421-427, 1981) supplemented with 0.1 mg/L BA, 3 g/L activated charcoal and 1.5% sucrose.

5        Somatic Embryogenesis and Regeneration

Anthers were isolated under sterile conditions and plated at a density of forty to fifty anthers per 9.0 cm diameter Petri dish and cultured at 28°C in the dark. Callus was induced on MSE. After sixty days, embryos were induced and then transferred to hormone-free HMG medium for differentiation. Torpedo-stage embryos were transferred 10 from HMG to MGC medium to promote embryo germination. Cultures were maintained in the dark at 26-28°C and transferred to fresh medium at three to four week intervals. Hypocotyls (elongated embryos) were transferred to rooting medium in baby food jars (five to eight embryos per jar). The embryos were grown at 25°C with a daily sixteen hour photoperiod to induce shoot and root formation. After root development plants were 15 transplanted to soil and placed in the greenhouse.

Maintenance and Propagation of Somatic embryos

Hypocotyls from elongated embryos that developed in HMG or MGC medium were cut into 3-4 mm pieces and placed onto MSE medium to promote the development of secondary embryogenic calli. The secondary embryogenic calli were then transferred 20 to HMG for differentiation and development of new hypocotyls. These secondary hypocotyls from HMG medium were then transferred to MSE medium to obtain a third cycle of embryogenic calli and hypocotyls. The fourth and fifth cycles of embryogenic calli were obtained in a similar manner. Alternatively, embryogenic calli developing from the anthers were propagated on MSE medium to produce sufficient young embryos 25 for transformation. All embryo cultures were transferred at twenty to thirty day intervals to fresh medium for maintenance.

Genes and Vectors

Three genetic constructs were used to genetically transform grape in this study (Fig. 2). *A. tumefaciens* strain C58Z707 containing either the binary plasmid pGA482GG 30 or pGA482G were used to transform the grape plants with GFLV-cp. The coat protein

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gene of a GFLV designated as 'Geneva' NY isolate CF57 was cloned and sequenced according to standard methods. The coat protein of grapevine fanleaf nepovirus is produced by posttranslational processing of the polyprotein by virus encoded proteinase. The coat protein gene, which is located on the 3' half of the RNA2 genome, does not 5 contain an ATG initiation codon. Oligonucleotide primers containing the NcoI site were therefore used to introduce the translatable initiation codon into a genetic construct. Two primer sets designed to flank the coat protein gene for PCR amplification were utilized according to standard methods. The primer set (P2:  
cgtcagTCTAGACCATGGTGAGAGGATTAGCTGGTAGAGGAG (SEQ ID NO: 3)  
10 and KSL95-10: ctgtaCCATGGTCTTTAAAGTCAGATACC (SEQ ID NO: 4)) was used to generate a translatable construct. To engineer a sense nontranslatable construct, we introduced an additional nucleotide (T) only three nucleotides downstream of the translational initiation codon (ATG) to make a frameshift mutation, as well as to create a stop codon. This was accomplished using the KSL95-10 (SEQ ID NO: 4) and KSL96-15  
15 (acgttaCCATGGTGAGAGGATTAGCTGGTAGA; SEQ ID NO: 5) primers. (Lower case letters are nonsense sequences which are utilized for effective restriction digestion. Underlined areas are restriction sites which were used for efficient cloning. Bold letters represent the stop codon which was used to engineer a sense nontranslatable construct.) The resulting amplified PCR products were treated with the restriction enzyme, NcoI, and  
20 cloned into the plant expression vector pEPT8. Sense or antisense orientation was determined using standard restriction mapping and PCR analysis making use of the positional 35S promoter-specific primer (KSL96-12:  
agtgcCTCGAGCAATTGAGACTTTCAACAA; SEQ ID NO: 6) and transgene  
primers. The expression cassette containing the transgene and plant transcriptional  
25 elements, 35S enhancers, 35S promoter, alfalfa mosaic virus RNA4 5' untranslated sequence and 35S terminator was subsequently cloned into the plant transformation vector pGA482G.

Transformation

Transformation protocols were modified from those described by Scorza and  
30 Cordts, (*Plant Cell Rep.* 14: 589-592, 1995; Krastanova et al., *Plant Cell Rep.* 24: 550-

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- 554, 1995). Overnight cultures of *Agrobacterium* strain C58Z707 were grown in LB medium at 28°C in a shaking incubator. Bacteria were centrifuged for five minutes at 5000 rpm (or 3000 rpm) and resuspended in MS liquid medium ( $OD_{600} = 0.4\text{--}1.0$ ). Callus with globular or heart-shaped embryos was immersed in the bacterial suspension for fifteen to thirty minutes, blotted dry, and transferred to HMG medium with or without acetosyringone (100  $\mu\text{M}$ ). The embryogenic calli were cocultivated with the bacteria for forty-eight hours in the dark at 28°C. Next, the plant material was washed in MS liquid plus cefotaxime (300 mg/ml) and carbenicillin (200 mg/ml) two to three times. The material was then transferred to HMG medium containing either 20 or 40 mg/L kanamycin, 300 mg/L cefotaxime, and 200 mg/L carbenicillin to select transgenic embryos. Alternatively, after forty-eight hours of cocultivation with *Agrobacterium*, embryogenic calli were transferred onto initiation MSE medium containing 25 mg/L kanamycin plus the same antibiotics listed above. All plant material was incubated continuously in the dark at 28°C. After growth on selection medium for three months, 10 embryos were transferred to HMG or MGC without kanamycin for development of hypocotyls. Embryos were then transferred to rooting medium without antibiotics. Nontransformed calli were grown in the same media with and without kanamycin to verify the efficiency of the kanamycin selection and the ability of the plant to regenerate in the presence of the antibiotic.
- 15

20           Analysis of transgenic plants

Transgenic plants were analyzed using standard assays GUS assay (Jefferson, *Plant Mol. Biol. Rep.* 5: 387-405, 1987), ELISA for NPTII detection (Cabanes-Bastos et al., *Gene* 77: 69-176, 1989), ELISA for cp detection (Clark et al., *J. Gen. Virol.* 34: 475-483), and PCR and Southern analysis (Ausubel et al., *infra*).

25           Isolation of Other Grape Nepovirus-CP Genes

Any grape nepovirus (for example, GFLV) isolate can serve as the nucleic acid source for the molecular cloning of a grape nepovirus coat protein (CP) gene. For example, isolation of a GFLV-CP gene involves the isolation of those DNA sequences which encode a protein exhibiting CP-associated structures, properties, or activities.

- 30           Based on the GFLV-CP nucleotide and amino acid sequences described herein (Fig. 1;

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SEQ ID NOS: 1 and 2), the isolation of additional GFLV-CP coding sequences is made possible using standard strategies and techniques that are well known in the art.

- In one particular example, the GFLV-CP sequences described herein may be used, together with conventional screening methods of nucleic acid hybridization 5 screening. Such hybridization techniques and screening procedures are well known to those skilled in the art and are described, for example, in Benton and Davis, *Science* 196: 180, 1977; Grunstein and Hogness, *Proc. Natl. Acad. Sci., USA* 72: 3961, 1975; Ausubel et al. (*supra*); Berger and Kimmel (*supra*); and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York. In one particular 10 example, all or part of the 'Geneva' isolate nucleotide sequence (described herein) may be used as a probe to screen a recombinant GFLV DNA library for genes having sequence identity to the coat protein gene of the 'Geneva' isolate. Hybridizing sequences are detected by plaque or colony hybridization according to standard methods, for example those described below.
- 15 Alternatively, using all or a portion of the amino acid sequence of the coat protein gene of the 'Geneva' isolate one may readily design GFLV-CP-specific oligonucleotide probes, including GFLV-CP degenerate oligonucleotide probes (i.e., a mixture of all possible coding sequences for a given amino acid sequence). These oligonucleotides may be based upon the sequence of either DNA strand and any 20 appropriate portion of the GFLV-CP sequence (Fig 1; SEQ ID NOS: 1 and 2). General methods for designing and preparing such probes are provided, for example, in Ausubel et al., 1996, *Current Protocols in Molecular Biology*, Wiley Interscience, New York, and Berger and Kimmel, *Guide to Molecular Cloning Techniques*, 1987, Academic Press, New York. These oligonucleotides are useful for GFLV-CP gene isolation, either 25 through their use as probes capable of hybridizing to GFLV-CP complementary sequences or as primers for various amplification techniques, for example, polymerase chain reaction (PCR) cloning strategies. If desired, a combination of different oligonucleotide probes may be used for the screening of a recombinant DNA library. The oligonucleotides may be detectably-labeled using methods known in the art and used to 30 probe filter replicates from a recombinant DNA library. Recombinant DNA libraries are

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prepared according to methods well known in the art, for example, as described in Ausubel et al. (*supra*), or they may be obtained from commercial sources.

Other sources of GFLV-CP sequences include those described in Brandt et al., *Arch. Virol.* 140: 157-164, 1995; Margis et al., *J. Gen. Virol.* 74: 1919-1926, 1993; Fuchs 5 et al., *J. Gen. Virol.* 955-962, 1989; Serghini et al., *J. Gen. Virol.* 71: 1433-1441, 1990; Bardonnec et al., *Plant Cell Rep.* 13: 357-360, 1994; Krastanova et al., *Plant Cell Rep.* 14: 550-554, 1995; Ritzenthaler et al., *J. Gen. Virol.* 72: 2357-2365, 1991; Mauro et al., *Plant Science*: 112: 97-106, 1995; and Sanchez et al., *Nucleic Acids Res.* 19: 5440, 1992. Once GFLV-CP sequence is identified, it is cloned according to standard methods and 10 used for the construction of plant expression vectors as described herein.

#### Construction of Plant Transgenes

Most preferably, a grape nepovirus coat protein (for example, a GFLV-CP) is expressed as a sense translatable or sense nontranslatable mRNA transcript or as an antisense mRNA transcript by a stably-transfected grape cell line or by a transgenic 15 grapevine or grapevine component. A number of vectors suitable for either stable or extrachromosomal transfection of plant cells, or for the establishment of transgenic plants are available to the public; such vectors are described in Weissbach and Weissbach (*Methods for Plant Molecular Biology*, Academic Press, 1989) and Gelvin et al. (*Plant Molecular Biology Manual*, Kluwer, Academic Publishers, 1990). Methods for constructing such cell lines are described in, for example, Weissbach and Weissbach 20 (*supra*), and Gelvin et al. (*supra*). Example of vectors useful for the expression of transgenes in grapevines are also described in Scorza et al. (*Plant Cell Rep.* 14: 589-592, 1995), Baribault et al. (*J. Expt. Bot.* 41: 1045-1049, 1990), Mullins et al. (*BioTechnology* 8: 1041-1045, 1990), Nakano et al. (*J. Expt. Bot.* 45: 649-656, 1994), Kikkert et al. (*Plant 25 Cell Rep.* 15: 311-316, 1995), Krastanova et al. (*Plant Cell Rep.* 1: 550-554, 1995), Scorza et al. (*Plant Cell Rep.* 14: 589-592, 1994), Scorza et al. (*J. Amer. Soc. Hort. Sci.* 121: 616-619, 1996), Martinelli et al. (*Theor Appl Genet.* 88: 621-628, 1994), and Legall et al. (*Plant Sci.* 102: 161-170, 1994).

Typically, plant expression vectors include (1) a cloned gene (for example, a 30 nucleic acid molecule which expresses a sense translatable, sense nontranslatable, or

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antisense grape nepovirus RNA) under the transcriptional control of 5' and 3' expression control sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (for example, one conferring inducible or constitutive, pathogen- or wound-induced, environmentally- or

- 5 developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

Once the desired grape nepovirus coat protein nucleic acid molecule is obtained as described above, it may be manipulated in a variety of ways known in the art.

- 10 For example, a GFLV-CP DNA sequence of the invention may, if desired, be combined with other DNA sequences in a variety of ways. The GFLV-CP DNA sequence may be employed with all or part of the gene sequences normally associated with the GFLV-CP. In its component parts, a DNA sequence encoding a GFLV-CP is combined in a DNA construct having a transcription initiation control region capable of promoting
- 15 transcription in a host grapevine cell.

In general, the constructs will involve regulatory regions functional in plants which provide for modified production of a GFLV-CP as discussed herein. For example, the sense nontranslatable sequence for a GFLV-CP or fragment thereof will be joined at its 5' end to a transcription initiation regulatory region, for example, such as a sequence naturally found in the 5' upstream region of a plant structural gene. Numerous transcription initiation regions are available which provide for constitutive or inducible regulation.

- 20
- For applications where developmental, cell, tissue, hormonal, or environmental expression is desired, appropriate 5' upstream non-coding regions are obtained from other genes, for example, from genes regulated during meristem development, seed development, embryo development, leaf development, stem development, or tendril development.

Regulatory transcript termination regions may also be provided in DNA constructs of this invention as well. Transcript termination regions may be provided by

25

the DNA sequence encoding the GFLV-CP or any convenient transcription termination

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region derived from a different gene source (for example, the NOS or 35S CaMV terminators). The transcript termination region will contain preferably at least 1-3 kb of sequence 3' to the structural gene from which the termination region is derived. Plant expression constructs having GFLV-CP as the DNA sequence of interest for expression  
5 (in either the antisense orientation or sense translatable or sense nontranslatable production of mRNA) may be employed with a wide variety of grapevines. Such genetically-engineered plants are useful for a variety of industrial and agricultural applications. Importantly, this invention is applicable to all grapevines or grapevine components, and will be readily applicable to any new or improved transformation or  
10 regeneration methods of grape.

The expression constructs include at least one promoter operably linked to at least one sense translatable or sense nontranslatable or antisense GFLV-CP sequence. An example of a useful plant promoter according to the invention is a caulimovirus promoter, for example, a cauliflower mosaic virus (CaMV) promoter. These promoters confer high  
15 levels of expression in most plant tissues, and the activity of these promoters is not dependent on virally encoded proteins. CaMV is a source for both the 35S and 19S promoters. In most tissues of transgenic plants, the CaMV 35S promoter is a strong promoter (see, for example, Odell et al., *Nature* 313: 810, 1985). The CaMV promoter is also highly active in monocots (see, for example, Dekeyser et al., *Plant Cell* 2: 591, 1990;  
20 Terada and Shimamoto, *Mol. Gen. Genet.* 220: 389, 1990). Moreover, activity of this promoter can be further increased (i.e., between 2-10 fold) by duplication of the CaMV 35S promoter (see for example, Kay et al., *Science* 236: 1299, 1987; Ow et al., *Proc. Natl. Acad. Sci., U.S.A.* 84: 4870, 1987; and Fang et al., *Plant Cell* 1: 141, 1989, and McPherson and Kay, U.S. Pat. No. 5,378,142).

25 Other useful plant promoters include, without limitation, the nopaline synthase (NOS) promoter (An et al., *Plant Physiol.* 88: 547, 1988), the octopine synthase promoter (Fromm et al., *Plant Cell* 1: 977, 1989), the rice actin promoter (Wu and McElroy, WO91/09948), the cyclase promoter (Chappell et al., WO96/36697), and the cassava vein mosaic virus promoter (Verdaguer et al., *Plant Mol. Biol.* 31: 1129-1139, 1996). Still  
30 other exemplary promoters useful in the invention include, without limitation, commelina

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yellow mottle virus promoter, sugar cane badna virus promoter, rice tungro bacilliform virus promoter, maize streak virus element, and wheat dwarf virus promoter.

- For certain applications, it may be desirable to produce the GFLV-CP sequence in an appropriate tissue, at an appropriate level, or at an appropriate 5 developmental time. For this purpose, there are an assortment of gene promoters, each with its own distinct characteristics embodied in its regulatory sequences, shown to be regulated in response to inducible signals such as the environment, hormones, and/or developmental cues. These include, without limitation, gene promoters that are responsible for heat-regulated gene expression (see, for example, Callis et al., *Plant 10 Physiol.* 88: 965, 1988; Takahashi and Komeda, *Mol. Gen. Genet.* 219: 365, 1989; and Takahashi et al. *Plant J.* 2: 751, 1992), light-regulated gene expression (for example, the pea *rbcS-3A* described by Kuhlemeier et al., *Plant Cell* 1: 471, 1989; the maize *rbcS* promoter described by Schäffner and Sheen, *Plant Cell* 3: 997, 1991; the chlorophyll a/b-binding protein gene found in pea described by Simpson et al., *EMBO J.* 4: 2723, 1985; 15 the Arabssu promoter; or the rice rbs promoter), hormone-regulated gene expression (for example, the abscisic acid (ABA) responsive sequences from the *Em* gene of wheat described by Marcotte et al., *Plant Cell* 1: 969, 1989; the ABA-inducible HVA1 and HVA22, and rd29A promoters described for barley and *Arabidopsis* by Straub et al., *Plant Cell* 6: 617, 1994 and Shen et al., *Plant Cell* 7: 295, 1995; and wound-induced 20 gene expression (for example, of *wunI* described by Siebertz et al., *Plant Cell* 1: 961, 1989), organ-specific gene expression (for example, of the tuber-specific storage protein gene described by Roshal et al., *EMBO J.* 6: 1155, 1987; the 23-kDa zein gene from maize described by Schernthaner et al., *EMBO J.* 7: 1249, 1988; or the French bean  $\beta$ -phaseolin gene described by Bustos et al., *Plant Cell* 1: 839, 1989), or pathogen-inducible 25 promoters (for example, PR-1, prp-1, or  $\beta$ -1,3 glucanase promoters, the fungal-inducible wirla promoter of wheat, and the nematode-inducible promoters, TobRB7-5A and Hmg-1, of tobacco and parsley, respectively).

Plant expression vectors may also optionally include RNA processing signals, e.g. introns, which have been shown to be important for efficient RNA synthesis and 30 accumulation (Callis et al., *Genes and Dev.* 1: 1183, 1987). The location of the RNA

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splice sequences can dramatically influence the level of transgene expression in plants. In view of this fact, an intron may be positioned upstream or downstream of a GFLV-CP sequence in the transgene to modulate levels of gene expression.

In addition to the aforementioned 5' regulatory control sequences, the  
5 expression vectors may also include regulatory control regions which are generally present in the 3' regions of plant genes (Thornburg et al., *Proc. Natl. Acad. Sci. U.S.A.* 84: 744, 1987; An et al., *Plant Cell* 1: 115, 1989). For example, the 3' terminator region may be included in the expression vector to increase stability of the mRNA. One such 10 terminator region may be derived from the PI-II terminator region of potato. In addition, other commonly used terminators are derived from the octopine or nopaline synthase 15 signals.

The plant expression vector also typically contains a dominant selectable marker gene used to identify those cells that have become transformed. Useful selectable genes for plant systems include genes encoding antibiotic resistance genes, for example, 15 those encoding resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin, or spectinomycin. Genes required for photosynthesis may also be used as selectable markers in photosynthetic-deficient strains. Finally, genes encoding herbicide resistance may be used as selectable markers; useful herbicide resistance genes include the *bar* gene 20 encoding the enzyme phosphinothricin acetyltransferase and conferring resistance to the broad spectrum herbicide BASTA® (Hoechst AG, Frankfurt, Germany).

In addition, if desired, the plant expression construct may contain a modified or fully-synthetic GFLV-CP sequence which has been changed to enhance the performance of the gene in plants.

It should be readily apparent to one skilled in the art of molecular biology, 25 especially in the field of plant molecular biology, that the level of gene expression is dependent, not only on the combination of promoters, RNA processing signals, and terminator elements, but also on how these elements are used to increase the levels of selectable marker gene expression.

#### Grapevine Transformation

30 Upon construction of the plant expression vector, several standard methods are

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- available for introduction of the vector into a plant host, thereby generating a transgenic plant. These methods include (1) Agrobacterium-mediated transformation (*A. tumefaciens* or *A. rhizogenes*) (see, for example, Lichtenstein and Fuller In: *Genetic Engineering*, vol 6, PWJ Rigby, ed, London, Academic Press, 1987; and Lichtenstein, C.P., and Draper, J., In: *DNA Cloning*, Vol II, D.M. Glover, ed, Oxford, IRI Press, 1985)), (2) the particle delivery system (see, for example, Gordon-Kamm et al., *Plant Cell* 2: 603 (1990); or Sanford et al. U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792,), (3) microinjection protocols (see, for example, Green et al., *supra*), (4) polyethylene glycol (PEG) procedures (see, for example, Draper et al., *Plant Cell Physiol.* 10: 23: 451, 1982; or for example, Zhang and Wu, *Theor. Appl. Genet.* 76: 835, 1988), (5) liposome-mediated DNA uptake (see, for example, Freeman et al., *Plant Cell Physiol.* 25: 1353, 1984), (6) electroporation protocols (see, for example, Gelvin et al., *supra*; Dekeyser et al., *supra*; Fromm et al., *Nature* 319: 791, 1986; Sheen *Plant Cell* 2: 1027, 1990; or Jang and Sheen *Plant Cell* 6: 1665, 1994), and (7) the vortexing method (see, for example, Kindle *supra*). The method of transformation is not critical to the invention. Any method which provides for efficient transformation may be employed. Some exemplary methods for transforming grapes are found in Scorza et al. (*Plant Cell Reports* 14: 589-592, 1995), Baribault et al. (*J. Expt. Bot.* 41: 1045-1049, 1990), Mullins et al. (*BioTechnology* 8: 1041-1045, 1990), Nakano et al. (*J. Expt. Bot.* 45: 649-656, 1994), Kikkert et al. (*Plant Cell Rep.* 15: 311-316, 1995), Krastanova et al. (*Plant Cell Rep.* 1: 550-554, 1995), Scorza et al. (*Plant Cell Rep.* 14: 589-592, 1994), Scorza et al. (*J. Amer. Soc. Hort. Sci.* 121: 616-619, 1996), Martinelli et al. (*Theor Appl Genet.* 88: 621-628, 1994), and Legall et al. (*Plant Sci.* 102: 161-170, 1994). As newer methods are available to transform crops or other host cells, they may be directly applied as well.
- Suitable plants for use in the practice of the invention include, but are not limited to, grapevines (for example, *Vitis spp.*, *Vitis spp.* hybrids, and all members of the subgenera *Euvitis* and *Muscadinia*), including scion or rootstock cultivars. Exemplary scion cultivars include, without limitation, those which are referred to as table or raisin grapes and those used in wine production such as Cabernet Franc, Cabernet Sauvignon, Chardonnay (for example, CH 01, CH 02, CH Dijon), Merlot, Pinot Noir (PN, PN Dijon),

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- Semillon, White Riesling, Lambrusco, Thompson Seedless, Autumn Seedless, Niagrara Seedless, and Seval Blanc. Other scion cultivars which can be used include those commonly referred to as Table or Raisin Grapes, such as Alden, Almeria, Anab-E-Shahi, Autumn Black, Beauty Seedless, Black Corinth, Black Damascus, Black Malvoisie,
- 5 Black Prince, Blackrose, Bronx Seedless, Burgrave, Calmeria, Campbell Early, Canner, Cardinal, Catawba, Christmas, Concord, Dattier, Delight, Diamond, Dizmar, Duchess, Early Muscat, Emerald Seedless, Emperor, Exotic, Ferdinand de Lesseps, Fiesta, Flame seedless, Flame Tokay, Gasconade, Gold, Himrod, Hunisa, Hussiene, Isabella, Italia, July Muscat, Khandahar, Katta, Kourgane, Kishmishi, Loose Perlette, Malaga, Monukka,
- 10 Muscat of Alexandria, Muscat Flame, Muscat Hamburg, New York Muscat, Niabell, Niagara, Olivette blanche, Ontario, Pierce, Queen, Red Malaga, Ribier, Rish Baba, Romulus, Ruby Seedless, Schuyler, Seneca, Suavis (IP 365), Thompson seedless, and Thomuscat. They also include those used in wine production, such as Aleatico, Alicante Bouschet, Aligote, Alvarelhao, Aramon, Baco blanc (22A), Burger, Cabernet franc,
- 15 Cabernet, Sauvignon, Calzin, Carignane, Charbono, Chardonnay, Chasselas dore, Chenin blanc, Clairette blanche, Early Burgundy, Emerald Riesling, Feher Szagos, Fernao Pires, Flora, French Colombard, Fresia, Furmint, Gamay, Gewurztraminer, Grand noir, Gray Riesling, Green Hungarian, Green Veltliner, Grenache, Grillo, Helena, Inzolia, Lagrein, Lambrusco de Salamino, Malbec, Malvasia bianca, Mataro, Melon, Merlot, Meunier,
- 20 Mission, Montua de Pilas, Muscadelle du Bordelais, Muscat blanc, Muscat Ottonel, Muscat Saint-Vallier, Nebbiolo, Nebbiolo fino, Nebbiolo Lampia, Orange Muscat, Palomino, Pedro Ximenes, Petit Bouschet, Petite Sirah, Peverella, Pinot noir, Pinot Saint-George, Primitivo di Gioa, Red Veltliner, Refosco, Rkatsiteli, Royalty, Rubired, Ruby Cabernet, Saint-Emilion, Saint Macaire, Salvador, Sangiovese, Sauvignon blanc,
- 25 Sauvignon gris, Sauvignon vert, Scarlet, Seibel 5279, Seibel 9110, Seibel 13053, Semillon, Servant, Shiraz, Souzao, Sultana Crimson, Sylvaner, Tannat, Teroldico, Tinta Madeira, Tinto cao, Touriga, Traminer, Trebbiano Toscano, Trouseau, Valdepenas, Viognier, Walschriesling, White Riesling, and Zinfandel.

Rootstock cultivars that are useful in the invention include, without limitation,

- 30 *Vitis rupestris* Constantia, *Vitis rupestris* St. George, *Vitis californica*, *Vitis girdiana*, *Vitis*

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*rotundifolia*, *Vitis rotundifolia* Carlos, Richter 110 (*Vitis berlandieri x rupestris*), 101-14 Millardet et de Grasset (*Vitis riparia x rupestris*), Teleki 5C (*Vitis berlandieri x riparia*), 3309 Couderc (*Vitis riparia x rupestris*), Riparia Gloire de Montpellier (*Vitis riparia*), 5BB Teleki (selection Kober, *Vitis berlandieri x riparia*), SO<sub>4</sub> (*Vitis berlandieri x rupestris*), 41B Millardet (*Vitis vinifera x berlandieri*), and 039-16 (*Vitis vinifera x Muscadinia*). Additional rootstock cultivars which can be used include Couderc 1202, Couderc 1613, Couderc 1616, Couderc 3309, Dog Ridge, Foex 33EM, Freedom, Ganzin 1 (A x R #1), Harmony, Kober 5BB, LN33, Millardet & de Grasset 41B, Millardet & de Grasset 420A, Millardet & de Grasset 101-14, Oppenheim 4 (SO4), Paulsen 775, Paulsen 1045, Paulsen 1103, Richter 99, Richter 110, Riparia Gloire, Ruggeri 225, Saint-George, Salt Creek, Teleki 5A, *Vitis rupestris Constantia*, *Vitis californica*, and *Vitis girdiana*.

In general, transfer and expression of transgenes in plant cells, including grape plants, are now routine practices to those skilled in the art, and have become major tools to carry out gene expression studies in plants and to produce improved plant varieties of agricultural or commercial interest.

#### Transgenic Grapevine Regeneration

Plant cells transformed with a plant expression vector can be regenerated, for example, from single cells, callus tissue, or leaf discs according to standard plant tissue culture techniques. It is well known in the art that various cells, tissues, and organs from almost any plant can be successfully cultured to regenerate an entire plant; such techniques are described, for example, in Vasil *supra*; Green et al., *supra*; Weissbach and Weissbach, *supra*; and Gelvin et al., *supra*.

In one particular example, a cloned sense nontranslatable GFLV-CP sequence (for example, a sense translatable or sense nontranslatable coat protein gene, or an antisense construct) construct (for example, a GFLV-CP sequence in the sense orientation having an out-of-reading frame ATG including a stop codon after the initiation codon) under the control of the 35S CaMV promoter and the nopaline synthase terminator and carrying a selectable marker (for example, kanamycin resistance) is transformed into *Agrobacterium*. Transformation of grapevine with vector-containing *Agrobacterium* is carried out as described by Scorza and Cordts. Putative transformants are selected after a

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few weeks on plant tissue culture media containing kanamycin. Kanamycin-resistant plant material is then placed on plant tissue culture media without hormones for root initiation.

Transgenic plants expressing the selectable marker are then screened for  
5 transmission of the transgene DNA by standard detection techniques as described above. Each positive transgenic plant and its transgenic progeny are unique in comparison to other transgenic plants established with the same transgene. Integration of the transgene DNA into the plant genomic DNA is in most cases random, and the site of integration can profoundly affect the levels and the tissue and developmental patterns of transgene  
10 expression. Consequently, a number of transgenic lines are usually screened for each transgene to identify and select plants with the most appropriate expression profiles.

Transgenic lines are evaluated for levels of transgene expression. Expression at the RNA level is determined initially to identify and quantitate expression-positive plants. Standard techniques for RNA analysis are employed and include Northern blot  
15 assays and nuclear run-off assays (see, for example, Ausubel et al., *supra*). The RNA-positive plants are then analyzed for resistance to GFLV infection using the methods described above. Transformed grapevines that express a sense nontranslatable GFLV-CP sequence having resistance to fanleaf disease relative to control plants are taken as being useful in the invention.

20 All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

What is claimed is:

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Claims

1. A method for selecting a transgenic grapevine or grapevine component having increased resistance to a fanleaf disease, said method comprising:
  - (a) transforming a grape plant cell with a grape nepovirus coat protein nucleic acid molecule or fragment thereof which is capable of being expressed in said plant cell;
  - 5 (b) regenerating a transgenic grapevine or grapevine component from the plant cell; and
  - (c) selecting a transgenic grapevine or grapevine component which expresses, at a low level, said nucleic acid molecule or fragment thereof, wherein said low level expression increases the resistance of the transgenic grapevine or grapevine component to said fanleaf disease.
- 10 2. The method of claim 1, wherein said nucleic acid molecule or fragment thereof is encoded by a transgene integrated into a transgenic grapevine genome.
- 15 3. The method of claim 1, wherein said nucleic acid molecule or fragment thereof is expressed in a sense orientation.
4. The method of claim 3, wherein said nucleic acid molecule or fragment thereof is expressed as a sense nontranslatable mRNA molecule.
5. The method of claim 1, wherein said nucleic acid molecule or fragment thereof is expressed in an antisense orientation.
- 20 6. The method of claim 1, wherein said grapevine is a member of the genus *Vitis*.
7. The method of claim 1, wherein said grapevine component is a somatic embryo.

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8. The method of claim 1, wherein said grapevine component is a scion.
9. The method of claim 1, wherein said grapevine component is a rootstock.
10. The method of claim 1, wherein said grape nepovirus coat protein nucleic acid molecule is from a grapevine fanleaf virus.
- 5 11. The method of claim 1, wherein further comprising a grape nepovirus coat protein nucleic acid molecule or fragment thereof having about 50% or greater sequence identity to SEQ ID NO: 1.
- 10 12. A substantially pure protein comprising an amino acid sequence having at least 97% amino acid identity to the amino acid sequence of the 'Geneva' isolate grape nepovirus coat protein of SEQ ID NO: 2.
13. The substantially pure protein of claim 12, wherein said protein comprises the amino acid sequence of the grape nepovirus coat protein of SEQ ID NO: 2.
14. The substantially pure protein of claim 12, wherein said protein has the amino acid sequence of the grape nepovirus coat protein of SEQ ID NO: 2 or a fragment thereof.
- 15 16. An isolated nucleic acid molecule encoding a recombinant protein comprising an amino acid sequence having at least 97% amino acid identity to the amino acid sequence of the 'Geneva' isolate grape nepovirus coat protein of SEQ ID NO: 2.
- 20 17. The isolated nucleic acid molecule of claim 15, wherein said protein comprises the amino acid sequence of SEQ ID NO: 2.
18. The isolated nucleic acid molecule of claim 15, wherein said protein has

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the amino acid sequence of SEQ ID NO: 2 or a fragment thereof.

18. The isolated nucleic acid molecule of claim 15, wherein said nucleic acid molecule is operably linked to an expression control region.

19. A vector comprising an isolated nucleic acid molecule encoding a recombinant protein comprising an amino acid sequence having at least 97% amino acid identity to the amino acid sequence of the 'Geneva' isolate grape nepovirus coat protein of SEQ ID NO: 2.

20. The vector of claim 19, wherein said nucleic acid molecule is position for expression of a sense translatable RNA transcript.

10 21. The vector of claim 19, wherein said nucleic acid molecule is position for expression of a sense nontranslatable RNA transcript.

22. The vector of claim 19, wherein said nucleic acid molecule is position for expression of an antisense RNA sequence.

23. A cell transformed with the vector of claim 19.

15 24. A transgenic plant or plant component, comprising the vector of claim 19.

25. The transgenic plant or plant component of claim 24, wherein said nucleic acid molecule is expressed in said plant as a sense translatable RNA transcript.

26. The transgenic plant or plant component of claim 24, wherein said 20 nucleic acid molecule is expressed in said plant as a sense nontranslatable RNA

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transcript.

27. The transgenic plant or plant component of claim 24, wherein said nucleic acid molecule is expressed in said plant as an antisense RNA transcript.

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## SEQ ID NO:1

1 gtgagttggatttagtggtagaggagtgatttatccctaaggattgccaggaaatagg  
 60 cactcacctaatacgaccatctcactaaataataggattctaacggccgttatcc  
 G L A G R G V I Y I P K D C Q A N R

SEQ ID NO:2

61 tacttgggcaccttaaatatacgagatatgttcagatttaagggtgtccagtacgaa  
 120 atgaaccctgtggaaatttatatgtctatactagatgtctaaaattcccacaggtcatgctt  
 Y L G T L N I R D M I S D F K G V Q Y E

121 aagtggataactgcaggattgtcatgcctactttagaatagtgttaggctacctgca  
 180 ttcacctattgacgtcctaatacgatcgatggatggaaatcttatcaacaatccgatggacgt  
 K W I T A G L V M P T F R I V V R L P A

181 aatgccttactggattgacgtgggtatgagcttcgtatgtttataaccggatagctgt  
 240 ttacggaaatgacctaactgcacccactactcgaaatgttgcataatggctatcgatca  
 N A F T G L T W V M S F D A Y N R I A S

241 agaattactgttagtgcggatcctgtatacactctgtcagtcccacattggcttatccat  
 300 tcttaatgacgtcacgcctagacatatgtgagacagtgcagggtgtacccatggataggta  
 R I T A S A D P V Y T L S V P H W L I H

301 cataagttggcacgttacatgtgaaatagactatggagaattgtgtggtcacgcctg  
 360 gtattcaaccctgtgcaaattgtacactttatgtataccctttaacacaccgtgcggat  
 H K L G T F T C E I D Y G E L C G H A M

361 tggtttaagtccacaaccttgagtctccgaggctacacttcacgtgtttaaacggcaac  
 420 accaaattcagggtgtggaaactcgaggctccgtgtgaagtgcacgaaattgcccgtt  
 W F K S T T F E S P R L H F T C L T G N

421 aacaaagagttggcggcagactggcaagctgtcgtagaactgtatgcggaaattgaaagag  
 480 ttgtttctcaaccgcgtctgaccgttcgacacgttgcatacgccttacccatctc  
 N K E L A A D W Q A V V E L Y A E L E E

481 gccacgtccttccttggaaaccaacttgggtttgacccagggtgtttaaatggtaaa  
 540 cggtgtcaggaaaggaaacccttgggtgaaaccaaaaactgggtccacgaaaattaccatt  
 A T S F L G K P T L V F D P G A F N G K

**FIG. IA**

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541 tttcaattcctgacttgcctcccatttcttgatctaacagccgttacggctttagg  
 600 aaggtaaggactgaacggggggaaaagaactagattgtcgcaatgccgagaatcc  
 F Q F L T C P P I F F D L T A V T A L R  
  
 601 agtactgggtaacgttaggacaagtccaaatgggtgtactaccaaggtataaatcta  
 660 tcatgacccgattgcaatcctgttcagggttaccaaccatgtatggttccatatgttagat  
 S T G L T L G Q V P M V G T T K V Y N L  
  
 661 aatagtactctcgtaggttatggaaatgggggtactattagaggaagggtgcac  
 720 ttatcatgagagactcaacataaaatccttacccctccatgataatctcctccacgtg  
 N S T L V S C I L G M G G T I R G R V H  
  
 721 atttgcgccaatcttctatagtattgtttatgggtttagttagtggaaacgggacc  
 780 taaacacgcggttagaagatatacataacaaaatacccaaacatcactcacccctgg  
 I C A P I F Y S I V L W V V S E W N G T  
  
 781 actatggattgaatgaactttcaaataatccccgggtgtatgttagaagaggacggaagt  
 840 tgatacctaacctacttggaaatggcccccacatacatcttcctgcctca  
 T M D W N E L F K Y P G V Y V E E D G S  
  
 841 tttgaagtcaaaatccgttctccatatcaccgaactcctgctagattgcttgcataaccaa  
 900 aaacctcagtttaggcaagaggatagtggcttggacgtctaaacgattgg  
 F E V K I R S P Y H R T P A R L L A N Q  
  
 901 agccagaggatatgagctctctgaatttctatgcaatagcaggacctatagctccgtcg  
 960 tcggtctccctatactcgagagactaaagatacgttatcgctcctggatatcgaggcagc  
 S Q R D M S S L N F Y A I A G P I A P S  
  
 961 ggtgaaactgcacgacttccatagtcgtcagattgtatgggtttagttagtgcgcggccagatctc  
 1020 ccacttgcgtgctgaaggatatcagcacgtctaactacttttagcacgcgggtctagag  
 G E T A R L P I V V Q I D E I V R P D L  
  
 1021 tctctgccaagtttgaagatgattactttgtatgggtttagttagtgcgcggccagatctc  
 1080 agagacgggttcaaaaacttctactaatgaaacatacccacctgaaaagtctcaagtgagaa  
 S L P S F E D D Y F V W V D F S E F T L

**FIG. IB**

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1081 gacaaagaagaatcgagattggttctcgccctttgacttacttcaaatacttgtaga  
 1140 ctgtttcttccttagctctaaccaggcgaagaaaactgaaatgaaatgttatgaacatct  
 D K E E I E I G S R F F D F T S N T C R

1141 gtgtctatggagaaaaatccgttgcgtatgattgtcatggattgcacagtggc  
 1200 cacagataccctcttttaggcaaacgcacgatactaacaacgtacctaactgttcaccg  
 V S M G E N P F A A M I A C H G L H S H

1201 gtattggacctcaaatttcaatggagtctgaacaccgaatttggcaagagcagcgggagc  
 1260 cataacctggagttaaagttacctcagacttgtggcttaaaccgttctgtcgccctcg  
 V L D L K F Q W S L N T E F G K S S G S

1261 attacaattacgaagctgggggtgataaagccacaggcttgatggcccttcttgtt  
 1320 taatgttaatgttcgaccacccactatttcgggtgtccgaacctacccggaaagaacacaa  
 I T I T K L V G D K A T G L D G P S C V

1321 ttcccataaaaaagctgggggaactgcagagtgttatttgcaggagca  
 1380 aagcggtatgtttcgacctccctgacgtctcaacaactaacccctaaacgtctcg  
 F A I Q K L E G T A E L L I G N F A G A

1381 aacccaaactctcatttctctctacagtcggatggcgattaaactagatcaagca  
 1440 ttgggtttgagagtaaagagagagatgtccacccatccgttaatttgcatttt  
 N P N S H F S L Y S R W M A I K L D Q A

1441 aagagtatcaaagtactccgcgtttgttaaacctcgccaggcttagttttatgga  
 1500 ttctcatagttcatgaggcgcaaaacacatttgcggatggcaggatccaaatcac  
 K S I K V L R V L C K P R P G F S F Y G

1501 agaaccagcttcccagtctaggatcttactttaaaa  
 1543 tcttggtcgaagggtcagatccccatagaatgaaatttt  
 R T S F P V \*

**FIG. IC**

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## Gene constructs used to transform grape

Gene	Construct
1.) FLcpST	B <sub>R</sub> — [NPTII] — [2enh] [35S] [AIMV] — GFLV-CP — [35St] — B <sub>L</sub>
2.) FLcpAS	B <sub>R</sub> — [NPTII] — [2enh] [35S] [AIMV] — GFLV-CP — [35St] — B <sub>L</sub>
3.) FLcp+GUS	B <sub>R</sub> — [NPTII] — [35S] [CMV] — GFLV-CP — [35St] — GUS — B <sub>L</sub>

FIG. 2

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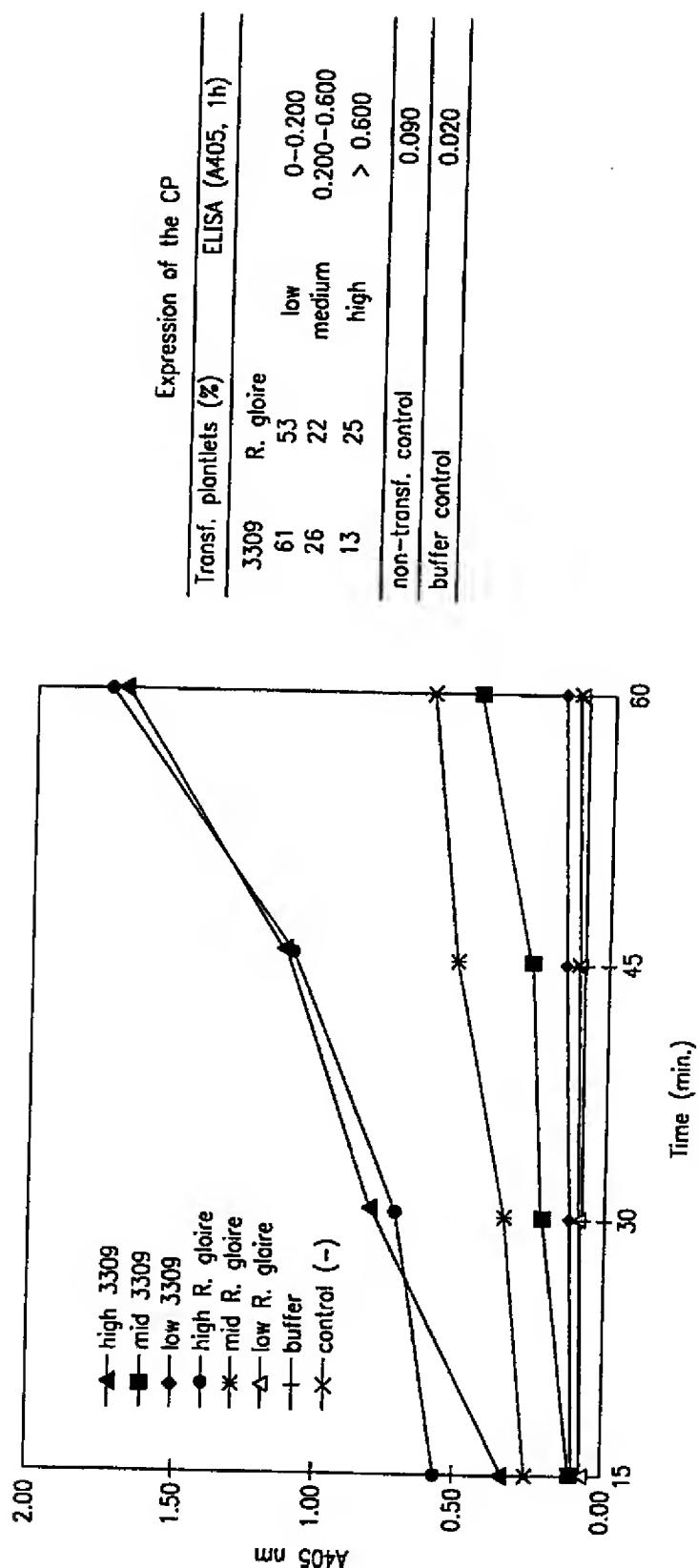


FIG. 3

**SEQUENCE LISTING**

<110> Cornell Research Foundation, Inc.

<120> NEPOVIRUS RESISTANCE IN GRAPEVINE

<130> 07678/023W02

<150> 60/060,384  
<151> 1997-09-29

<160> 6

<170> FastSEQ for Windows Version 3.0

<210> 1

<211> 1542

<212> DNA

<213> Grapevine Fanleaf Virus Coat Protein Gene

<220>

<221> CDS

<222> (7)...(1518)

<400> 1

```

gtgagt gga tta gct ggt aga gga gtg att tat atc cct aag gat tgc 48
      Gly Leu Ala Gly Arg Gly Val Ile Tyr Ile Pro Lys Asp Cys
      1           5             10

```

```

cag gca aat agg tac ttg ggc acc tta aat ata cga gat atg atc tca 96
Gln Ala Asn Arg Tyr Leu Gly Thr Leu Asn Ile Arg Asp Met Ile Ser
   15          20          25          30

```

gat ttt aag ggt gtc cag tac gaa aag tgg ata act gca gga tta gtc 144  
 Asp Phe Lys Gly Val Gln Tyr Glu Lys Trp Ile Thr Ala Gly Leu Val  
 35 40 45

atg cct act ttt aga ata gtt gtt agg cta cct gca aat gcc ttt act 192  
Met Pro Thr Phe Arg Ile Val Val Arg Leu Pro Ala Asn Ala Phe Thr  
50 55 60

gga ttg acg tgg gtg atg agc ttc gat got tat aac cgg ata gct agt 240  
Gly Leu Thr Trp Val Met Ser Phe Asp Ala Tyr Asn Arg Ile Ala Ser  
65 70 75

aga att act gct agt gcg gat cct gta tac act ctg tca gtc cca cat 288  
Arg Ile Thr Ala Ser Ala Asp Pro Val Tyr Thr Leu Ser Val Pro His  
80 85 90

tgg ctt atc cat cat aag ttg ggc acg ttt aca tgt gaa ata gac tat Trp Leu Ile His His Lys Leu Gly Thr Phe Thr Cys Glu Ile Asp Tyr 95 100 105 110	336
gga gaa ttg tgt ggt cac gcc atg tgg ttt aag tcc aca acc ttt gag Gly Glu Leu Cys Gly His Ala Met Trp Phe Lys Ser Thr Thr Phe Glu 115 120 125	384
tct ccg agg cta cac ttc acg tgc tta acg ggc aac aac aaa gag ttg Ser Pro Arg Leu His Phe Thr Cys Leu Thr Gly Asn Asn Lys Glu Leu 130 135 140	432
gcg gca gac tgg caa gct gtc gta gaa ctg tat gcg gaa ttg gaa gag Ala Ala Asp Trp Gln Ala Val Val Glu Leu Tyr Ala Glu Leu Glu Glu 145 150 155	480
gcc acg tcc ttc ctt ggg aaa cca act ttg gtt ttt gac cca ggt gct Ala Thr Ser Phe Leu Gly Lys Pro Thr Leu Val Phe Asp Pro Gly Ala 160 165 170	528
ttt aat ggt aaa ttt caa ttc ctg act tgc cct ccc att ttc ttt gat Phe Asn Gly Lys Phe Gln Phe Leu Thr Cys Pro Pro Ile Phe Phe Asp 175 180 185 190	576
cta aca gcc gtt acg gct ctt agg agt act ggg cta acg tta gga caa Leu Thr Ala Val Thr Ala Leu Arg Ser Thr Gly Leu Thr Leu Gly Gln 195 200 205	624
gtc cca atg gtt ggt act acc aag gta tac aat cta aat agt act ctc Val Pro Met Val Gly Thr Thr Lys Val Tyr Asn Leu Asn Ser Thr Leu 210 215 220	672
gtg agt tgt att tta gga atg gga ggt act att aga gga agg gtg cac Val Ser Cys Ile Leu Gly Met Gly Gly Thr Ile Arg Gly Arg Val His 225 230 235	720
att tgt gcg cca atc ttc tat agt att gtt tta tgg gtt gtt agt gag Ile Cys Ala Pro Ile Phe Tyr Ser Ile Val Leu Trp Val Val Ser Glu 240 245 250	768
tgg aac ggg acc act atg gat tgg aat gaa ctt ttc aaa tat ccc ggg Trp Asn Gly Thr Thr Met Asp Trp Asn Glu Leu Phe Lys Tyr Pro Gly 255 260 265 270	816
gtg tat gta gaa gag gac gga agt ttt gaa gtc aaa atc cgt tct cca Val Tyr Val Glu Glu Asp Gly Ser Phe Glu Val Lys Ile Arg Ser Pro 275 280 285	864
tat cac cga act cct gct aga ttg ctt gct aac caa agc cag agg gat Tyr His Arg Thr Pro Ala Arg Leu Leu Ala Asn Gln Ser Gln Arg Asp 290 295 300	912

atg agc tct ctg aat ttc tat gca ata gca gga cct ata gct ccg tcg Met Ser Ser Leu Asn Phe Tyr Ala Ile Ala Gly Pro Ile Ala Pro Ser 305                   310                   315	960
ggt gaa act gca cga ctt cct ata gtc gtg cag att gat gaa atc gtg Gly Glu Thr Ala Arg Leu Pro Ile Val Val Gln Ile Asp Glu Ile Val 320                   325                   330	1008
cgc cca gat ctc tct ctg cca agt ttt gaa gat gat tac ttt gta tgg Arg Pro Asp Leu Ser Leu Pro Ser Phe Glu Asp Asp Tyr Phe Val Trp 335                   340                   345                   350	1056
gtg gac ttt tca gag ttc act ctt gac aaa gaa gaa atc gag att ggt Val Asp Phe Ser Glu Phe Thr Leu Asp Lys Glu Glu Ile Glu Ile Gly 355                   360                   365	1104
tct cgc ttc ttt gac ttt act tca aat act tgt aga gtg tct atg gga Ser Arg Phe Asp Phe Thr Ser Asn Thr Cys Arg Val Ser Met Gly 370                   375                   380	1152
gaa aat ccg ttt gct gct atg att gct tgt cat gga ttg cac agt ggc Glu Asn Pro Phe Ala Ala Met Ile Ala Cys His Gly Leu His Ser Gly 385                   390                   395	1200
gta ttg gac ctc aaa ttt caa tgg agt ctg aac acc gaa ttt ggc aag Val Leu Asp Leu Lys Phe Gln Trp Ser Leu Asn Thr Glu Phe Gly Lys 400                   405                   410	1248
agc agc ggg agc att aca att acg aag ctg gtg ggt gat aaa gcc aca Ser Ser Gly Ser Ile Thr Ile Thr Lys Leu Val Gly Asp Lys Ala Thr 415                   420                   425                   430	1296
ggc ttg gat ggg cct tct tgt gtt ttc gcc ata caa aag ctg gag gga Gly Leu Asp Gly Pro Ser Cys Val Phe Ala Ile Gln Lys Leu Glu Gly 435                   440                   445	1344
act gca gag ttg ttg att ggg aat ttt gca gga gca aac cca aac tct Thr Ala Glu Leu Ile Gly Asn Phe Ala Gly Ala Asn Pro Asn Ser 450                   455                   460	1392
cat ttc tct ctc tac agt cgg tgg atg gcg att aaa cta gat caa gca His Phe Ser Leu Tyr Ser Arg Trp Met Ala Ile Lys Leu Asp Gln Ala 465                   470                   475	1440
aag agt atc aaa gta ctc cgc gtt ttg tgt aaa cct cgt cca ggc ttt Lys Ser Ile Lys Val Leu Arg Val Leu Cys Lys Pro Arg Pro Gly Phe 480                   485                   490	1488
agt ttt tat gga aga acc agc ttc cca gtc tagggtatct tactttaaaa Ser Phe Tyr Gly Arg Thr Ser Phe Pro Val	1538

495	500	
gacc		1542
<pre> &lt;210&gt; 2 &lt;211&gt; 504 &lt;212&gt; PRT &lt;213&gt; Grapevine Fanleaf Virus Coat Protein </pre>		
<pre> &lt;400&gt; 2 Gly Leu Ala Gly Arg Gly Val Ile Tyr Ile Pro Lys Asp Cys Gln Ala  1           5          10          15 Asn Arg Tyr Leu Gly Thr Leu Asn Ile Arg Asp Met Ile Ser Asp Phe  20          25          30 Lys Gly Val Gln Tyr Glu Lys Trp Ile Thr Ala Gly Leu Val Met Pro  35          40          45 Thr Phe Arg Ile Val Val Arg Leu Pro Ala Asn Ala Phe Thr Gly Leu  50          55          60 Thr Trp Val Met Ser Phe Asp Ala Tyr Asn Arg Ile Ala Ser Arg Ile  65          70          75          80 Thr Ala Ser Ala Asp Pro Val Tyr Thr Leu Ser Val Pro His Trp Leu  85          90          95 Ile His His Lys Leu Gly Thr Phe Thr Cys Glu Ile Asp Tyr Gly Glu 100         105         110 Leu Cys Gly His Ala Met Trp Phe Lys Ser Thr Thr Phe Glu Ser Pro 115         120         125 Arg Leu His Phe Thr Cys Leu Thr Gly Asn Asn Lys Glu Leu Ala Ala 130         135         140 Asp Trp Gln Ala Val Val Glu Leu Tyr Ala Glu Leu Glu Ala Thr 145         150         155         160 Ser Phe Leu Gly Lys Pro Thr Leu Val Phe Asp Pro Gly Ala Phe Asn 165         170         175 Gly Lys Phe Gln Phe Leu Thr Cys Pro Pro Ile Phe Phe Asp Leu Thr 180         185         190 Ala Val Thr Ala Leu Arg Ser Thr Gly Leu Thr Leu Gly Gln Val Pro 195         200         205 Met Val Gly Thr Thr Lys Val Tyr Asn Leu Asn Ser Thr Leu Val Ser 210         215         220 Cys Ile Leu Gly Met Gly Gly Thr Ile Arg Gly Arg Val His Ile Cys 225         230         235         240 Ala Pro Ile Phe Tyr Ser Ile Val Leu Trp Val Val Ser Glu Trp Asn 245         250         255 Gly Thr Thr Met Asp Trp Asn Glu Leu Phe Lys Tyr Pro Gly Val Tyr 260         265         270 Val Glu Glu Asp Gly Ser Phe Glu Val Lys Ile Arg Ser Pro Tyr His 275         280         285 Arg Thr Pro Ala Arg Leu Leu Ala Asn Gln Ser Gln Arg Asp Met Ser 290         295         300 Ser Leu Asn Phe Tyr Ala Ile Ala Gly Pro Ile Ala Pro Ser Gly Glu 305         310         315         320 Thr Ala Arg Leu Pro Ile Val Val Gln Ile Asp Glu Ile Val Arg Pro </pre>		

325	330	335
Asp Leu Ser Leu Pro Ser Phe Glu Asp Asp Tyr Phe Val Trp Val Asp		
340	345	350
Phe Ser Glu Phe Thr Leu Asp Lys Glu Glu Ile Glu Ile Gly Ser Arg		
355	360	365
Phe Phe Asp Phe Thr Ser Asn Thr Cys Arg Val Ser Met Gly Glu Asn		
370	375	380
Pro Phe Ala Ala Met Ile Ala Cys His Gly Leu His Ser Gly Val Leu		
385	390	395
Asp Leu Lys Phe Gln Trp Ser Leu Asn Thr Glu Phe Gly Lys Ser Ser		
405	410	415
Gly Ser Ile Thr Ile Thr Lys Leu Val Gly Asp Lys Ala Thr Gly Leu		
420	425	430
Asp Gly Pro Ser Cys Val Phe Ala Ile Gln Lys Leu Glu Gly Thr Ala		
435	440	445
Glu Leu Leu Ile Gly Asn Phe Ala Gly Ala Asn Pro Asn Ser His Phe		
450	455	460
Ser Leu Tyr Ser Arg Trp Met Ala Ile Lys Leu Asp Gln Ala Lys Ser		
465	470	475
Ile Lys Val Leu Arg Val Leu Cys Lys Pro Arg Pro Gly Phe Ser Phe		
485	490	495
Tyr Gly Arg Thr Ser Phe Pro Val		
500		

&lt;210&gt; 3

&lt;211&gt; 42

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer

&lt;400&gt; 3

cgtcagtcta gaccatggtg agaggattag ctggtagagg ag

42

&lt;210&gt; 4

&lt;211&gt; 30

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer

&lt;400&gt; 4

ctgtaccatg gtctttaaa gtcagatacc

30

&lt;210&gt; 5

&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

<220>

<223> Primer

<400> 5

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33

<210> 6

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer

<400> 6

agtgcctctcg agcaattgag acttttcaac aa

32

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/20272

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 410, 419, 430, 430.1, 468; 800/278, 280, 288, 295, 298, 301

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Agricola, Capus

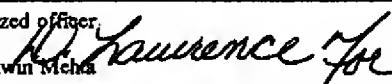
**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KIKKERT et al. Transgenic Plantlets of 'Chancellor' Grapevine (Vitis sp.) From Biostatic Transformation of Embryogenic Cell Suspensions. Plant Cell Rep. 1996, Vol. 15, pages 311-316, especially pages 312 and 316.	1-3, 6-8, 23-25
X	KRASTANOVA et al. Transformation of Grapevine Rootstocks with the Coat Protein Gene of Grapevine Fanleaf Nepovirus. Plant Cell Rep. 1995. Vol. 14. pages 550-554, see whole document	1-3, 6-11 -----
Y	BARDONNET et al. Protection Against Virus Infection in Tobacco Plants Expressing the Coat Protein of Grapevine Fanleaf Nepovirus. Plant Cell Rep. 1994. Vol. 13. pages 357-360, see whole document.	15-20, 23-25
X		23-25 -----
--		15-20
Y		

Further documents are listed in the continuation of Box C.  See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance		
"B" earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"A"	document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
03 DECEMBER 1998	29 DEC 1998

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer  Ashwin Mehta Telephone No. (703) 308-0196
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/20272

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SERGHINI et al. RNA2 of Grapevine Fanleaf Virus: Sequence Analysis and Coat Protein Cistron Location. J. Gen. Virol. 1990. Vol. 71, pages 1433-1441, especially pages 1433-1448.	15, 16 -----
--		
Y		1-3, 6-11, 17-20, 23-25

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/20272

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
  
  
  
2.  Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
  
  
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
  
  
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-3, 6-11, 15-20, 23-25

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/US98/20272

**A. CLASSIFICATION OF SUBJECT MATTER:**  
**IPC (6):**

A01H 1/00, 5/00, 5/08; C12N 5/00, 5/04, 5/10, 5/14, 15/00, 15/82

**A. CLASSIFICATION OF SUBJECT MATTER:**  
**US CL :**

435/69.1, 410, 419, 430, 430.1, 468; 800/278, 280, 288, 295, 298, 301

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-3, 6-11, 15-20, 23-25, drawn to a first method of selecting a fanleaf disease-resistant transgenic grape plant, comprising transformation with a first product comprising a vector comprising an isolated nepovirus coat protein (CP) gene, and wherein the gene expresses a sense, translatable RNA transcript; the transformed plant.

Group II, claim(s) 1, 2, 4, 6-11, 15-19, 21, 23, 24, 26, drawn to a second method for selecting a fanleaf disease-resistant grape plant, comprising expressing a second product encoding a sense, non-translatable transcript of the CP gene.

Group III, claim(s) 1, 2, 5-11, 15-19, 22-24, 27, drawn to a third method of selecting a fanleaf disease-resistant grape plant, comprising expressing a third product expressing an antisense transcript of the CP gene.

Group IV, claims 12-14, drawn a fourth product, the protein of SEQ ID NO:2.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The gene of Group I expresses a different product from that of Groups II and III, and is not required by any of the other groups. The same is true of the genes of Groups II and III. Groups II and III also do not require the product of Group IV. The product of Group IV does not require the methods of Groups I-III, and can be chemically synthesized.